Published in final edited form as:
Biochim Biophys Acta. 2016 October ; 1858(10): 2390–2400. doi:10.1016/j.bbamem.2016.02.037.

Lipid Interaction Sites on Channels, Transporters and Receptors: Recent Insights from Molecular Dynamics Simulations

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Abstract

Lipid molecules are able to selectively interact with specific sites on integral membrane proteins, and modulate their structure and function. Identification and characterisation of these sites is of importance for our understanding of the molecular basis of membrane protein function and stability, and may facilitate the design of lipid-like drug molecules. Molecular dynamics simulations provide a powerful tool for the identification of these sites, complementing advances in membrane protein structural biology and biophysics. We describe recent notable biomolecular simulation studies which have identified lipid interaction sites on a range of different membrane proteins. The sites identified in these simulation studies agree well with those identified by complementary experimental techniques. This demonstrates the power of the molecular dynamics approach in the prediction and characterization of lipid interaction sites on integral membrane proteins.

Keywords

Lipid-protein interaction; Lipid binding site; MD simulation; Cholesterol; Cardiolipin; PIP_2

1 Introduction

Cells are separated from their environment and compartmentalised by membranes. These barriers are composed of lipid bilayers (with the various lipid species distributed asymmetrically between the two leaflets of the bilayer), into which proteins are embedded. Parallel advances in lipidomics [1] and in the structural biology of membrane proteins [2, 3] over the past decade have revealed some of the complexities of the composition of cell membranes. Thus, the structures of ca. 1500 membrane proteins have been determined (http://blanco.biomol.uci.edu/mpstruc/) and the cellular lipidome is estimated to contain 40,000 lipid species (http://www.lipidmaps.org/data/structure/) [4]. The lipidome of membranes varies according to cell age, metabolic state, stage in cell cycle, organelle, and spatial location; resulting in a complex protein-lipid interactome. In addition to providing a bilayer environment, it is increasingly appreciated that the function of embedded proteins

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For BBA Biomembranes, BBAMEM-16-23 (Special Issue)

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can be modulated by interactions with this complex lipid mixture [5–12]. Of particular interest, an emerging feature present within the protein-lipid interactome is that certain lipid molecules can selectively bind to specific sites on integral membrane proteins, and modulate both their structure and their function [13].

As recently reviewed [14], we now possess over 100 structures of membrane proteins containing electron density interpreted as bound lipid molecules. Structural identification of specific lipid binding sites aids our mechanistic understanding of lipid modulation of protein function, such as in the case of Kir2.2 and PIP_2 [15]. Identification of sites of allosteric modulation on proteins is also of interest for the assessment of protein druggability [16]. The majority of membrane protein structures containing bound lipid molecules were solved using x-ray crystallography. In many cases such structures have been obtained from crystals grown in the presence of detergent. It is likely that the lipids observed represent a biased sample of tight binding lipids, and in some cases the molecular identity of the observed electron density corresponding to detergent and/or lipid may be uncertain. This may change as more membrane protein structures are determined using crystals obtained from lipidic cubic phases [17] which better approximate a native membrane environment.

Molecular dynamics simulations allow membrane protein structures to be computationally re-embedded into lipid bilayers, and their dynamic interactions with surrounding lipid molecules to be characterised [18]. A number of recent simulation studies probing lipid interactions have identified specific lipid binding sites. These sites show good agreement with those identified from a range of structural studies. A number of other, presumably weaker, binding sites can also be resolved. Whilst these weaker sites may not always be observed by x-ray crystallography, there are a number of other biophysical techniques which allow us to probe lipid interactions with membrane proteins, including e.g. fluorescence spectroscopy [19], EPR [20], NMR [21], and mass spectrometry [13]. These techniques provide further points of reference for simulation studies membrane protein/lipid interactions.

Within this review article we survey recent simulation studies which identify lipid interaction sites on membrane proteins. We focus on specific binding of lipids to defined sites on membrane proteins. We also focus on channels, receptors, and transporters, for which there are functional and structural data on the biological importance of lipid/protein interactions. Overall we find molecular dynamics simulations to have strong predictive power and to be well-suited for identification of these sites. Additionally the simulation approach provides a means for further characterisation of the identified sites, for instance by estimations of lipid binding affinities [22–24], as well as enabling functional insight into mechanisms of lipid modulation [25–27].

1.1 Lipid modulation of membrane protein function

The functions of a range of membrane proteins are known to be modulated by their lipid environment, including potassium channels [6, 7], receptor tyrosine kinases (RTKs) [8], G-protein coupled receptors (GPCRs) [9, 10], solute transporters such as BetP [5] and the ADP/ATP carrier [28], redox proteins such as cytochrome c oxidase [29], and certain P-type ATPases [30]. Such lipid modulation can influence several different aspects of protein

function, including effects on the activity of a membrane protein, modulating protein-protein interactions, and altering cellular localisation by sequestering a protein to spatially defined regions of a membrane. In certain cases, a lipid may represent a native ligand for the protein rather than an allosteric modulator, as is the case for the sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) group of lipid-activated GPCRs [31].

In a number of cases structural, biophysical and functional assays have been combined to provide a detailed picture of lipid modulation. For example, this is the case for eukaryotic inward rectifying potassium ion (Kir) channels. Functional assays revealed Kir channels to be dependent on the presence of the anionic lipid phosphatidyl inositol-4,5-bisphosphate (PIP_2) for activation. Subsequently, simulation studies [25, 26] and crystal structures [15] revealed four specific PIP_2 binding sites and enabled the mechanism of PIP_2 channel modulation to be structurally rationalised (See Section 2.1.2). In other cases careful biochemical analysis has revealed a functional dependence on certain lipid species, but the mechanism of modulation remains unclear. This is the case for the epidermal growth factor receptor (EGFR/ErbB1) which is a single-pass transmembrane receptor known to be modulated by an array of lipids, including PIP species and the glycolipid GM3 [8]. However exactly how these lipid species control receptor activity remains unclear, with proposals including an influence of receptor dimerization propensity, direct conformational stabilization and orientation effects, and larger scale lipid-induced clustering of the receptor.

1.2 Lipid interaction sites on integral membrane proteins

Lipids interact with membrane proteins *via* multiple modes. The presence of integral membrane proteins may induce formation of a lipid ‘annulus’ around the protein. Due to interactions with the protein, lipids within this annulus exhibit decreased motional freedom compared to their non-interacting bulk counterparts, and are detectable by EPR [32, 33]. This immobilizing effect of the protein may extend beyond the first shell of directly interacting annular lipids, leading to further outer shells with a lesser extent of lipid immobilisation, as suggested by MD simulations [34, 35]. In addition certain lipid species may bind to specific sites on the membrane protein surface – often described as ‘non-annular’ lipids. Binding may be driven by formation of physicochemical interactions between the lipid and protein surface, as well as by complementary geometry, for instance ‘slotting’ of lipid molecules into ‘grooves’ on the protein surface [36] or binding at the interface between subunits [37]. Binding sites may tightly coordinate the lipid [15], or act to cause weaker and more dynamic localisation [38]. Efforts have been made to describe general features of lipid binding sites and sequence interaction motifs, such as for cholesterol [39] and cardiolipin [40].

1.3 Biomolecular simulation approaches for lipid binding site identification

Molecular dynamics (MD) simulations provide a powerful tool to characterise the dynamics and interactions of membrane proteins with surrounding lipid molecules [18]. However, the computational cost of the simulations is such that length scales beyond microseconds are not currently readily accessible [41], especially for extended systems containing multiple membrane proteins. This has prompted the development of more approximate coarse-grained (CG) representations of membrane lipids and proteins in MD simulations [42, 43] in

which groups of atoms are represented as single particles (Figure 1). Reducing the number of particles in the system reduces the computational demand involved in running the simulation and thus allows access to longer time and length scales, with the caveat that the level of approximation made in a given CG model has to be matched to the underlying biological interactions being probed. CG simulations can thus allow significantly enhanced lipid exploration of the protein surface and candidate binding sites, whilst sacrificing the finer detail of lipid-protein interactions. These approximations may be reconciled to some degree by conversion of the endpoint of a CG system back to atomistic detail [44, 45] and subsequently running an atomistic simulation to assess the validity of the CG system arrangement; so called (serial) multiscale modelling [46]. The MARTINI CG force field has been most widely applied in the field of protein-lipid interactions. We note that CG simulations may now extend to hundreds of microseconds [23], and contain many hundreds of protein molecules [47], whilst atomistic simulations of individual proteins may reach tens of microseconds duration using high performance computing resources [48].

The structure of a membrane protein used as initial input for MD simulations may be from X-ray diffraction, cryoelectron microscopy, or NMR. If the 3D structure of the protein is not known experimentally, in some cases a model may be built by modelling [35]. The membrane protein is then embedded into a lipid bilayer. This may be achieved either by self-assembly simulations [49] in which short simulations are run to allow the spontaneous formation of a bilayer around an integral membrane protein, or by a number of methods which insert a membrane protein into a pre-assembled bilayer [50–52]. Advances in lipid parameterisation [53], along with a growing appreciation of the *in vivo* compositional complexity of lipid membranes [54] is leading to simulations of proteins in complex bilayers containing multiple lipid species. At the simplest level such mixed bilayers may contain two lipid species, while at their most complex they may provide approximations of *in vivo* plasma membrane composition [55, 56]. Such mixed lipid systems allow us to address competition between different lipid species for interaction with a given protein, in addition to providing a better approximation of lipid-lipid interactions which are linked to, and may influence, protein-lipid interactions. Such mixed lipid systems can now be routinely assembled in CG [55, 56]. Simulations of mixed lipid systems are also becoming more common at the atomistic level, utilising a number of recent tools (e.g. [57, 58] including as the *Membrane Builder* extension of the CHARMM-GUI [59] to facilitate automated construction of complex bilayers in all atom detail. Some of the equilibration and sampling problems arising from timescale limitations being combated by increases in processing power and algorithm efficiency, as well as the ability to reversibly convert between CG and atomistic levels (Figure 1) [44, 45]. Thus in the latter approach the membrane protein of interest may first be simulated in a mixed lipid bilayer using CG methods to equilibrate the system and make an assessment of how lipids interact on extended timescales, before converting the system to atomistic detail to further refine and characterise the observed lipid-protein interactions. This multiscale approach has been successfully used to identify lipid binding sites on a number of membrane proteins [23, 25, 26, 60].

Potential lipid binding sites may also be identified by docking calculations, using e.g. AutoDock [61]. However these methods do not generally take into account the membrane environment in which the interactions occur. Thus protein/lipid configurations identified by

docking may require refinement by subsequent molecular dynamics simulation. Combined use of molecular docking and simulation has enabled identification of lipid binding sites on several types of membrane protein, including e.g. cholesterol interactions with Cys-loop receptors [62, 63] and with Kir channels [22].

2 Characterisation of lipid interaction sites: a digest of recent simulation studies

2.1 Channels

Lipids modulate the function of a number of channels. For example, the canonical bacterial potassium channel KcsA requires bound anionic lipid molecules for full activity, and both crystallographic [64] and simulation studies [65] have previously indicated the presence of a binding site for an anionic lipid molecule located between two arginine sidechains at the subunit/subunit interface of the trimeric channel protein. More recent studies have focussed on two species of lipid thought to regulate a number of channels in mammalian cell membranes, namely PIP_2 and cholesterol.

2.1.2 Inward rectifying potassium (Kir) channels—Kir channels are tetrameric integral membrane proteins controlling the selective permeation of K^+ ions across cell membranes. They have critical involvement in molecular processes ranging from control of the resting membrane potential to regulation of insulin secretion in pancreatic β cells [66]. Of particular interest Kir channels have a requirement for PIP_2 for maximal activation [6, 67] which may be considered as lipid agonism of these channels [68].

The interaction of PIP_2 with Kir channels has been explored using combined CG and atomistic simulations [25]. Three structures of Kir channels were simulated: KirBac1.1, a Kir3.1-KirBac1.3 chimera, and a homology model of Kir6.2. These structures were initially converted to CG representation and embedded into POPC bilayers, each of which contained 4 PIP_2 lipid molecules within the inner leaflet of the bilayer. Analysis of time-averaged density maps for PIP_2 revealed preferential localisation to a single site, present on all four subunits. The same site was observed for all three Kir channels. The binding site was formed by a cluster of basic and amphipathic aromatic residues at the interface between subunits. Once bound, PIP_2 lipids did not dissociate from the identified sites even during extended (5 μs) CG simulations, suggesting tight binding. Conversion of CG snapshots of the channel with bound PIP_2 lipids to atomistic models followed by short simulations enabled assessment of binding site interactions. Key residues seen to form contacts with the PIP_2 lipid head group in the simulations had previously been implicated in PIP_2 interactions with Kir2.1 [6, 69, 70]. This was not the case for residues seen in the simulations to form contacts with the lipid tail regions, which formed more transient interactions with the protein and were therefore not suggested to be major binding determinants.

Subsequent to this study, a high resolution X-ray structure of Kir2.2 in complex with four short chain (dioctanoyl) PIP_2 lipids was determined [15], revealing binding sites and interactions in good agreement with the simulation-based predictions [25]. This is despite the caveat that the structures used for simulations employed homology models and

eukaryotic-prokaryotic chimeras. Further studies [26] extended the comparison by multiscale simulations of the PIP_2 bound structure (PDB: 3SPI[15]), as well as other Kir2.2 X-ray structures including the apo state (PDB: 3JYC[71]), and a phosphatidic acid bound state (PDB: 3SPC[15]). This study provided a 'like for like' comparison of PIP_2 binding to Kir2.2, supporting the sites initially identified *via* simulations of other Kir subtypes. These multiscale simulations showed unambiguously that PIP_2 lipids bound at the same four sites identified in the crystal structure (Figure 2). The good agreement between multiple simulation and experimental approaches is suggestive of the accuracy of the binding site predictions, as well as the utility of multiscale simulations in their identification.

Kir channels have also been shown to be regulated by cholesterol [72, 73], in a stereospecific fashion suggestive of the existence of a specific cholesterol binding site on the channel proteins [7] [74]. An integrated molecular docking and simulation approach has been used to investigate cholesterol binding to Kir2.1 [22], leading to the identification of two cholesterol binding sites. The validity of the sites was tested by mutagenesis and electrophysiology, and showed good agreement with the MD predictions. Docking and simulation studies were also used to compare predicted cholesterol interactions of bacterial (KirBac) and mammalian Kir channels [73]. Overall, these two studies suggest a consensus cholesterol binding region in the cytoplasmic half of the TM domains of Kir channels with the ligand also interacting with residues on the slide helix located at the membrane surface. For a recent comprehensive review of cholesterol interactions with membrane proteins as studied by molecular dynamics see [75].

2.1.2 Pentameric ligand gated ion-channels—The pentameric ligand gated ion-channels (pLGICs) are a major class of neurotransmitter receptor [76]. Both the nicotinic acetylcholine receptor (nAChR) and the y-aminobutyric acid receptor ($GABA_AR$) are sensitive to membrane cholesterol [11, 12, 77], and the nAChR also shows functional sensitivity to anionic lipids [78] and to membrane hydrophobic thickness [79]. The interactions of cholesterol with the nAChR [62, 80] and the $GABA_AR$ [63] have both been explored using docking and simulations. In a study by Brannigan et al., [62] docking calculations were performed to screen the nAChR *Torpedo* cryo-EM structure (PDB: 2BG9) for possible cholesterol binding sites [62]. A total of three sites per subunit were identified (15 for the whole protein). These sites were buried both within grooves on the protein surface, and more deeply within non-membrane exposed pockets. A more recent study [81] identified the presence of cholesterol interaction motifs within the transmembrane domain of nAChR, which co-localised with a number of the sites suggested by Brannigan et al., as well as a number of additional solvent exposed sites. Interestingly the sites identified in these computational studies corresponded to gaps in the original cryo-EM structure (PDB: 2BG9). The possible role of cholesterol at the identified sites was addressed by 25 ns atomistic simulations both in the presence and the absence of bound cholesterol [62]. In the absence of cholesterol the binding sites were initially filled with water. During the simulation water was expelled and the structure was seen to collapse. In contrast in the presence of bound cholesterol the original conformation was more faithfully maintained, and contacts between the pore and agonist binding domain proposed to be critical for gating were seen to be maintained. The results overall suggested 15 cholesterol molecules bind to the pentameric

nAChR structure and act to provide structural integrity to the receptor. Interactions of nAChR with phosphatidic acid (PA) have also been explored by simulations [80], which suggested potential differences in the interaction modes of anionic PA and zwitterionic lipids, although the relatively short duration (10 ns) of the simulations precludes more detailed comparisons.

In a second study, cholesterol interactions with the related $GABA_AR$ were explored [63]. In lieu of a high resolution structure of $GABA_AR$, the authors built a homology model based on GluCl (PDB: 3RHW [82]) and used this structure for initial protein coordinates within simulations. Five equivalent cholesterol binding pockets positioned between subunits of the pentameric receptor were identified by docking (Figure 3). Atomistic simulations were performed starting from a configuration with cholesterol docked into these sites. Cholesterol was seen to undergo some reorientation within all five sites. During a 200 ns simulation the cholesterol molecules at two sites were seen to dissociate within 20 ns, but were subsequently seen to rebind at around 80-110 ns with similar but presumably slightly more favourable binding modes. The remaining three cholesterol molecules remained bound. The general stability of cholesterol and the rebinding events observed during the simulations are supportive of the validity of the sites suggested from docking. A second model of cholesterol bound to $GABA_AR$ was built based on the ivermectin binding sites in the GluCl (ivermectin is a relatively hydrophobic ligand) crystal structure [82]. The binding pockets in this model were identical to those seen from docking and spontaneous rebinding events during atomistic simulation, though some differences in cholesterol orientation were observed. Thus the different approaches seemed to converge in terms of cholesterol interactions. Comparison of the behaviour of the protein in the cholesterol-bound and the apo state simulations suggested that in the absence of cholesterol the pore radius of the channel decreased, whereas with bound cholesterol the channel showed an increased tendency to adopt a more open conformation. This suggests that cholesterol may in part exert its channel modulating properties *via* direct binding and stabilisation of an open conformation of the $GABA_AR$.

2.1.3 Mechanosensitive channels—

Mechanosensitive channels open in response to an increase in tension (i.e. stretching) of the membrane. A recent combined structural, biophysical and computational study has revealed the role of bound lipid molecules in mechanosensing by the *E. coli* MscS channel [27]. X-ray diffraction and simulation together identified lipid tails bound inside pockets formed by the TM helices. Significantly the number of lipid acyl chains occupying the pockets decreased upon channel opening (Figure 4). This suggested an activation mechanism whereby an increase in membrane tension perturbed lipid binding within the pocket, thus destabilizing the closed state and thus promoting channel opening.

2.2 G protein coupled receptors

G protein coupled receptors (GPCRs) are a large superfamily of integral membrane proteins composed of seven transmembrane helices [83]. The complexities of the interactions of GPCRs with cholesterol and other lipids are starting to be unravelled [84]. Cholesterol has been suggested to modulate various aspects of GPCR biology including stability [9, 10],

oligomeric organisation [85], and ligand binding activity [86, 87]. Cholesterol molecules have been found co-crystallised in a number of GPCR X-ray structures [88–91], suggesting possible modulation by direct binding. Molecular simulation has been applied to identify lipid binding sites on a range of GPCRs including the β_2-adrenergic receptor (β_2AR) [92–94], the β_1-adrenergic receptor (β_1AR) [95], the Serotonin$_{1A}$ [96] and Serotonin$_{2A}$ receptors [97], the A_{2A} adenosine receptor [24], Rhodopsin [98, 99], the Cannabinoid 2 (CB2) receptor [100], and the S1P1 receptor [47].

Cholesterol binding sites have been identified on the A_{2A} adenosine receptor by long timescale all-atom MD simulations [24] and by X-ray crystallography [90]. In a study by Lee and Lyman [24], two independent 800 ns all-atom simulations of the adenosine-bound structure (PDB: 2YDO) were performed in a PC:cholesterol lipid bilayer. Over the course of the simulations cholesterol molecules bound to three distinct sites on the receptor. Once bound, a single cholesterol molecule occupied each site for the duration of the simulation. However despite these sites remaining occupied, the binding modes of cholesterol were seen to be dynamic. The simulation data were used to estimate cholesterol interaction energies at each site *via* an inverse Boltzmann approach, yielding energies of the order of kT, suggesting cholesterol is only weakly associated with the receptor. Concomitant to this study an X-ray structure of the A_{2A} adenosine receptor was published with three co-crystallised cholesterol molecules (Figure 5) [90]. Both the simulation and crystallographic studies [24, 90] agreed on site II. The differences in sites I and III between the crystal structure and simulations may reflect the relatively weak and dynamic nature of the cholesterol interactions, as well as sampling constraints.

CG simulations provide a means to significantly extend sampling of cholesterol/GPCR interactions. Thus, Sengupta et al. explored cholesterol binding to the Serotonin$_{1A}$ receptor [96] using CG simulations of up to 80 μs duration with the GPCR embedded in a PC:cholesterol bilayer. A number of sites were identified within both leaflets. On the basis of their lipid contact dynamics these sites were predicted to have a relatively weak propensity to bind cholesterol. Interestingly one site co-localised with a cholesterol consensus motif (CCM), and another with a cholesterol recognition amino acid consensus (CRAC) motif. However the significance of such motifs in general remains uncertain. These sites showed differences with those seen for the closely related Serotonin$_{2A}$ receptor [97], raising the question of how to best evaluate and validate simulation-based predictions.

A subsequent study utilized comparable CG simulations to investigate cholesterol interactions with the β_2AR [93]. These simulations were performed in bilayers containing two β_2AR molecules, allowing an exploration of cholesterol influence on GPCR dimerization. In the absence of cholesterol, protein dimerization was observed *via* an interface involving TM4 and TM5 from each monomer. Titration of increasing amounts of cholesterol into the lipid bilayer and subsequent simulation led to dissolution of this interface and emergence of a new interface involving helices TM1 and TM2 from each monomer. The TM4+TM5 and TM1+TM2 interfaces for β_2AR identified by CG simulation are reminiscent of those seen in atomistic simulations of β_1AR [95]. Computation of cholesterol density maps and cholesterol contact dynamics led to identification of three binding sites on the β_2AR in the outer leaflet, and four within the inner leaflet [101]. One of

these sites was formed on TM4 where it formed interactions with residues of a CCM motif. The binding modes at this site were dynamic, as seen for other GPCRs [24, 96]. Notably cholesterol was seen at this position in a crystal structure of β_2AR [102]. In the cholesterol-containing bilayers this binding site became occupied by the sterol, leading to disruption of the involvement of TM4 in the TM4+TM5 interface. These results suggest that cholesterol may modulate dimerization of the β_2AR *via* direct competition between lipid-protein and protein-protein interactions. This provides an example of the use of molecular simulations to explore effects of lipid binding on protein oligomerization and organization within membranes. These aspects have also been explored in large scale simulations of rhodopsin embedded in single lipid-species bilayer containing tens of protein molecules [103–105].

Recent large simulations of the S1P1 receptor in a plasma membrane model have provided further insights into the possible influence of lipid interactions on GPCR oligomerisation [47]. CG simulations of a 140 x 140 nm^2 patch of membrane (Figure 6) containing 144 copies of the S1P1 receptor and with a lipid composition mimicking that of a mammalian plasma membrane (extracellular leaflet PC:PE:Sph:GM3:Chol = 40:10:15:10:25; intracellular PC:PE:PS:PIP_2:Chol = 10:40:15:10:25) were run for 10 μs. Analysis of the simulations revealed transient formation of S1P1 dimers, trimers, and higher order oligomers. Interactions of cholesterol and of PIP_2 with the GPCR were observed (Figure 6). Detailed examination of the S1P1 dimers observed in the simulation suggested that cholesterol may help to mediate the protein-protein interactions.

Simulations have also been used to explore how anionic phospholipids may modulate β_2AR function [92]. From an extensive (0.25 ms) set of all-atom simulations it was possible to observe an anionic lipid, phosphatidylglycerol (PG), entering the core of the activated β_2AR laterally *via* an opening between the cytoplasmic portions of helices TM6 and TM7 (Figure 7). Once bound the PG molecule formed electrostatic interactions with the protein which inhibited the formation of the ionic lock, a key interaction thought to stabilise the inactive state of the receptor. Entry of the PG lipid thus led to an increase in stability of the active state of the transmembrane domain, providing a testable mechanism which may explain the experimental observation that anionic lipids can enhance the activity of certain GPCRs [106] including the β_2AR [107].

A number of other examples of identification of lipid binding sites on GPCRs are available [47, 94, 95, 98, 99]. These include long timescale atomistic simulations of the β_1AR[95] and CB2 receptors [100], and multiscale simulations of rhodopsin [98, 99]; as well as a recent study on possible effects of omega-3 fatty acids on oligomerization of A2A adenosine and of dopamine D2 receptors in the context of neuropsychiatric disease [108]. For a recent detailed review of the application of molecular simulations to explore GPCR-cholesterol interactions see [38].

2.3 Other receptors: receptor tyrosine kinases

A second major superfamily of membrane receptors are those which have a single membrane spanning helix, and which form functional dimers in cell membranes. These include the receptor tyrosine kinases (RTKs) [109] and also a number of other families including e.g. integrins [110]. These receptors are characterised by extensive extracellular

domains and single pass transmembrane helices. Many crystal structures are known for extracellular and intracellular domains, but a high resolution structure of an intact RTK remains elusive. A number of NMR structures of TM helix dimers from RTKs are known [111–114].

Experimental studies have shown that lipid interactions can modulate RTK activation, especially of the EGFR [8, 115]. There have been a number of simulation studies of: (i) models of the intact EGFR receptor in mixed lipid bilayers [116, 117]; and of (ii) the transmembrane helix of the EGFR and its dimers [35, 118, 119]. There has also been a recent study combining experiments and simulations to explore lipid interactions of the EphA2 receptor transmembrane region and ectodomain, and their influence on the conformation and orientation of the ectodomain relative to the membranes [120].

Recently we undertook a systematic comparative study of lipid interactions with models of the transmembrane helix plus juxtamembrane region (TM+JM) of all 58 human RTKs [35]. This revealed conservation of the interactions of the JM region with PIP_2 (Figure 8). Furthermore, these conserved interactions were seen to induce local bilayer reorganisation and anionic lipid clustering (Figure 8C) seen previously for the TM+JM regions of the gp130 cytokine receptor [34]. This behaviour is likely to extend to other single transmembrane domain proteins besides RTKs and related receptors. However, it is of especial interest for RTKs as PIP_2 lipids have been suggested to modulate the activity and cellular distribution of certain members of the family ([8, 121, 122] by interactions with the TM-JM domain.

This comparative simulation study demonstrated that simulations can now be used to compare protein/lipid interactions across whole families of membrane proteins (as has also proved possible for e.g. aquaporins [123]). Conservation of such interactions across a family of proteins is an indicator of their likely biological importance.

2.4 Transporter proteins

Lipids have been shown to play a critical role in the function of a number of solute transporters, and structural studies have revealed lipid binding sites on several transporter proteins [5]. Simulation based approaches have made a recent valuable contribution to identification and characterisation of sites on transporters including the ADP/ATP carrier [124], the UraA H^+-Uracil symporter [60], LacY [125], SERCA, the canonical P-type ATPase [126], and the dopamine transporter [127]

2.4.1 ADP/ATP carrier (AAC/ANT)—The ADP/ATP carrier (AAC/ANT) is the best studied member of the mitochondrial carrier family (MCF) of transporters, which enable the exchange of solutes across mitochondrial inner membranes [128]. In common with other MCF transporters, AAC/ANT consists of six transmembrane helices arranged around a central cavity, with three amphipathic matrix helices positioned perpendicular to the membrane normal. AAC/ANT requires the presence of cardiolipin for interactions with other proteins [28, 129], and the presence of cardiolipin is a requirement for reconstitution and crystallization of this protein. Simulations in a simple PC bilayer revealed three binding sites which coincide with the crystallographic locations of three quasi-equivalent cardiolipin

(CL) binding sites [124]. Recently we have explored in more detail interactions with cardiolipin by combined CG and atomistic simulations (*Rouse et al., unpublished data*). Multiple CG simulations of microscecond duration of bovine AAC/ANT (PDB: 1OKC) embedded in a PC:PE:CL lipid bilayer (molar ratio 6:6:1) were conducted to assess cardiolipin interactions with the transporter. In all five simulations cardiolipin molecules were seen to bind into three 'grooves' on the protein between the amphipathic matrix helices. These three sites were in excellent agreement with previous X-ray structures containing co-crystallised cardiolipin molecules [36, 130, 131] (Figure 9). Simulation of a recently published structure of yeast AAC2 (PDB: 4C9G[131]) revealed the same three cardiolipin binding sites. This conservation of binding sites reinforces their likely biological significance. A parallel independent CG simulation study identified the same three sites on bovine and yeast AAC/ANT (*Duncan et al., unpublished data*), and showed the bound cardiolipin molecules remained stable during subsequent 20 ns atomistic simulation. The reproduction of these same three sites between multiple crystal structures, and two independent simulation studies is strongly suggestive of their biological significance. Indeed, cardiolipin is thought to serve a role in mitochondrial membranes as a 'glue' or 'molecular filler' in mediating certain protein-protein interactions. These studies of cardiolipin interactions with the AAC/ANT transporter protein in the inner mitochondrial membrane nicely complement landmark studies of on cardiolipin interactions with components of the electron transport chain (namely cytochrome bc1 [23, 132] and cytochrome c oxidase [133]), and suggest how simulations of lipid-protein interactions might aid development of models of larger scale organization [134–136] of the mitochondrial inner membrane [137].

2.4.2 UraA H^+-Uracil symporter—The *Escherichia coli* UraA H^+-Uracil symporter is a member of the nucleobase-ascorbate transporter (NAT) family of transporters [138] which use proton and Na^+ gradients to enable uptake of xanthine, uric acid, and uracil, as well as vitamin C in mammals [138, 139]. A recent MD study explored lipid interactions of the bacterial UraA transporter (PDB: 3QE7) in a bilayer, [60] the lipid composition of which mimicked that of the bacterial inner membrane, containing PE (75 mol %), PG (20 mol %) and CL (5 mol %). CG simulations of up to 10 μs duration revealed an enrichment of the anionic lipids PG and CL within the lipid annulus. In particular, CL exhibited preferential interactions with the transporter at three sites (Figure 10). A periplasmic site was seen to be positioned in the vicinity of the transport pathway. Given CL may act as a proton donor and acceptor at physiological pH, this suggests a putative proton-donating role for this cardiolipin site. Analysis of protein-lipid contact patterns during the simulation showed cardiolipin molecules at each site interacted predominantly *via* electrostatic interactions between negatively charged cardiolipin headgroups and basic sidechains, whilst in common with observations from other simulation studies [23, 25] the tail groups exhibited more dynamic interactions. The possible importance of interactions seen in identified lipid binding sites may be tested *via in silico* mutagenesis of binding site residues. Using this approach sidechains critical for cardiolipin binding were identified [60]. No lipid molecules have been seen in X-ray structures of UraA, which was crystallised in the presence of a detergent (n-nonyl-β-D-glucopyranoside), one molecule of which was observed bound in the crystal structure in the centre of the protein on the presumed transport pathway of the solute. This

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suggests possible weak association of cardiolipin with UraA, which may dissociate during detergent treatment.

2.4.3 Ca^{2+} ATPase: SERCA—SERCA is a canonical P-type ATPase which enables ATP-driven reuptake of Ca^{2+} ions into the endo or sarcoplasmic reticulum during calcium signalling. Enrichment of cholesterol in ER membranes of macrophages has been shown to inhibit SERCA2b [30], and lipid-like drugs such as thapsigargin modulate SERCA. Further, phospholipids and other hydrophobic compounds have been observed in x-ray crystal structures [140, 141]. In a study by Autzen et al., thirty independent CG simulations of SERCA embedded in a PC:cholesterol bilayer (molar ratio 10:1) were conducted to address possible cholesterol binding and modulation of SERCA [126]. Mapping the frequency of cholesterol contacts for each residue of the protein onto the 3D structure revealed two major cholesterol binding pockets (Figure 11), one of which co-localized to a position known to bind sarcolipin, a small single TM helix protein which modulates SERCA activity. However, from the location of cholesterol within these sites and comparison of its exchange dynamics to bulk phospholipids it has proved difficult to rationalise how cholesterol binding may alter SERCA activity. Consideration of shared chemical features of thapsigargin and cholesterol lead to an expectation that cholesterol might bind in the thapsigargin binding pocket, but this was not observed in the simulations, in which the pocket instead was occupied by a phospholipid. A series of 100 ns all-atom simulations initiated from cholesterol manually positioned in the thapsigargin binding pocket (based on thapsigargin bound X-ray structure, PDB: 2C8K) did *not* result in a single stable configuration over the simulated time course, and instead exhibited a very dynamic cholesterol pose. This suggests that simulations may be used to rule out possible binding modes for lipids, although further experimental and computational studies will be needed to explore this in more detail.

2.5 Other membrane proteins

A number of simulation studies not described in detail in this review have also addressed lipid interaction sites of membrane proteins. These include phospholipid binding to LacY [125], Kv channels [142], aquaporins [123, 143], the fungal lipid scrambalase TMEM [124], and the dopamine transporter [127]; cardiolipin binding to respiratory chain complexes [23, 132, 133]; and cholesterol binding to rhodopsin [98, 99], VDAC [144], β_1AR [95] and β_2AR [94].

3 Summary and Conclusions

From a number of studies it is evident that molecular dynamics simulations enable us to identify lipid binding and/or interaction sites on integral membrane proteins, and in particular on a number of channels, receptors, and transporters. Molecular simulations provide a valuable complement to experimental approaches, especially X-ray diffraction and mass spectrometry in allowing us to analyse membrane protein/lipid interactions in detail. MD simulations can not only identify interaction sites but also may provide details of selectivity and can be applied to explore competition effects by including multiple lipid species in simulations [55, 56]. Furthermore, the energetics of lipid binding may be estimated, as has been seen for e.g. Kir-cholesterol [22] and Kir-PIP_2 interactions [145], A_{2A}

adenosine receptor-cholesterol interactions [24], and cytochrome bc1-cardiolipin interactions [23]. Simulations also enable comparison of lipid interactions with different conformational states of a protein, providing insights into function, e.g. for mechanosensitive ion channels [27].

Simulations of large ensembles of proteins in mixed lipid bilayers are starting to provide insights into protein/lipid interactions in membrane models which take increased account of the lipid compositional complexity of membranes *in vivo* [54]. Recent examples of complex lipid mixtures containing large ensembles of protein include simulations of the S1P1 receptor [47], the bacterial outer membrane proteins OmpF and BtuB [146], the membrane envelope of the influenza virion [147], mitochondrial respiratory chain complexes [137], and of the A_{2A} adenosine and dopamine D2 receptors embedded in membranes with either a 'healthy' (i.e. high omega-3 fatty acid content) or a 'disease state' (i.e. a low omega-3 fatty acid content) lipid composition [108]. Such investigations benefit enormously from continuing developments in the MARTINI coarse-grained force field [148, 149], especially for complex mixtures of lipids [53, 55] which allows binding sites to be identified simply by random diffusion of lipid molecules in large systems with multiple copies of a given membrane protein. This approach is not routinely possible yet in atomistic detail due to the need for extended (multi-microsecond) simulations on large systems and the concomitant computational demands. Rather, atomistic simulations may be coupled with CG or docking methods, using these approaches to initially survey the protein surface for candidate lipid interaction sites, before applying atomistic simulations to evaluate and refine these sites.

In parallel with developments in CG simulations there have also been ongoing advances in atomistic parameters for lipids (e.g. [150–156]). Of particular importance is the development of accurate parameters for a greater diversity of lipids [157], including e.g. phosphatidyl inositols [158, 159], and e.g. bacterial lipopolysaccharide models [160].

As the number of membrane protein structures continues to increase (see http://blanco.biomol.uci.edu/mpstruc/) there are considerable opportunities for systematic and comparative analysis of lipid-protein interactions across all membrane proteins of known structure. In parallel there have also been ongoing developments in enabling high throughput simulations for membrane proteins [105, 161]. With the emergence of high-throughput technologies for studying lipid-protein interactions experimentally [162], such simulation approaches will have increasing scope in complementing structural and biophysical studies. In particular, it will be interesting to incorporate computational tools to identify lipid binding and interaction sites within existing simulation pipelines and databases such as MemProtMD [124]. Such automation would provide a larger scale survey of the existing database of membrane protein structures, which will enable general patterns and trends in lipid interactions to be identified.

Acknowledgements

Research in M. S. P. S.'s group is funded by the BBSRC (BB/L002558/1), and the Wellcome Trust (WT092970). G.H. is funded by the MRC and the University of Oxford. We thank Dr. Anna Duncan and other members of the M.S.P.S laboratory for fruitful discussions. Thanks to Dr. Chris Neale and Professor Angel E. García for simulation structures of β_2AR used in the table of contents graphic.

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Abbreviations

MD	molecular dynamics
CG	coarse-grained
NMR	nuclear magnetic resonance
ESR	electron spin resonance
EM	electron microscopy
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
PIP_2	phosphatidylinositol-4,5-bisphosphate
Kir	inward rectifying potassium ion channel
nAChR	nicotinic acetylcholine receptor
$GABA_AR$	y-aminobutyric acid receptor
GPCR	G-protein coupled receptor
β_2AR	β_2-adrenergic receptor
ANT/AAC	ADP/ATP carrier
SERCA	sarco/endoplasmic reticulum Ca^{2+} ATPase.

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Highlights

- Lipid molecules can selectively interact with specific sites on membrane proteins
- Lipid binding can modulate protein structure and function
- Molecular dynamics (MD) simulations provide a powerful tool for site identification
- MD has been used to identify sites on a wide range of membrane proteins

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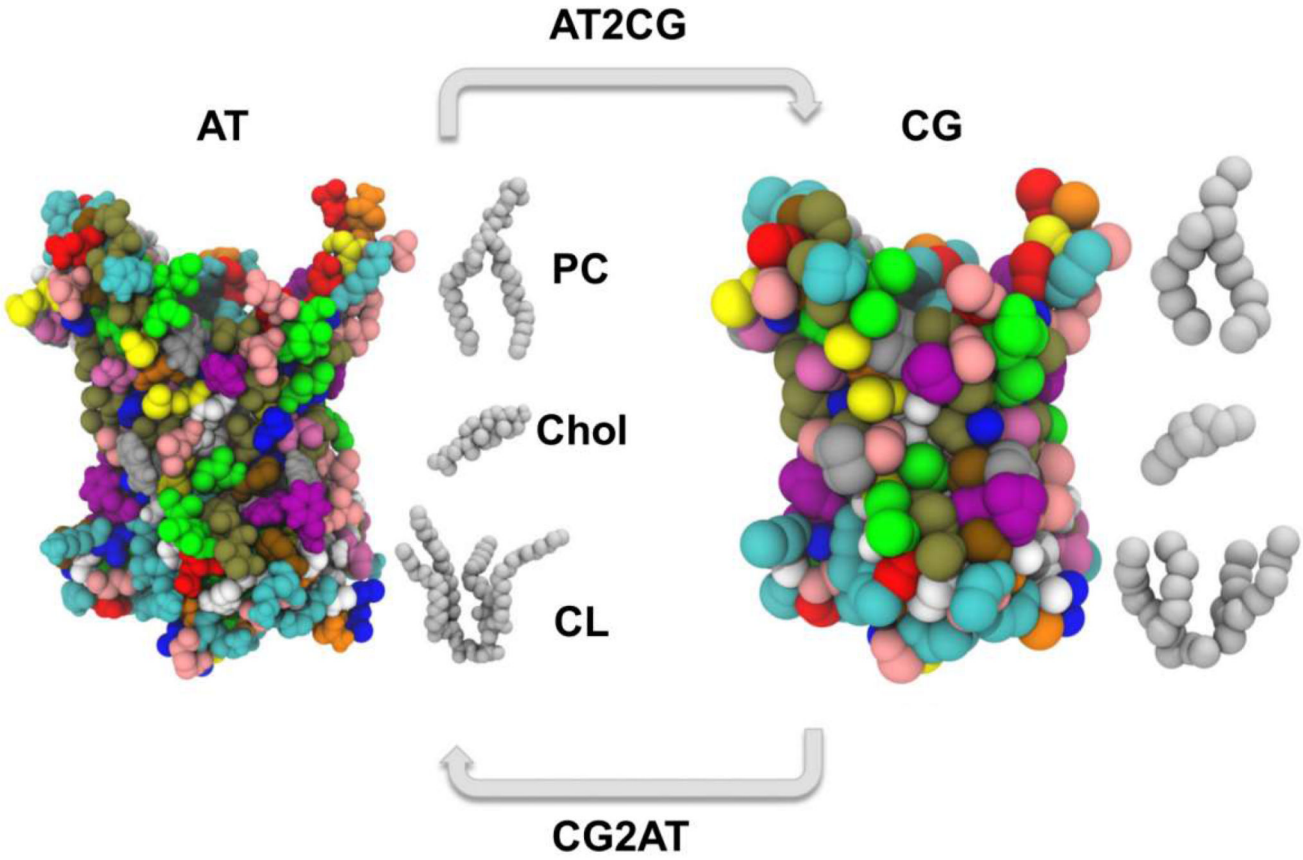

Figure 1.

Schematic of a multiscale approach to modelling and simulation of lipid interactions with an integral membrane protein. The ADP/ATP carrier (ANT1/AAC1; PDB: 1OKC [36]) is depicted as spheres coloured by residue type, at both the atomistic (left) and CG (right) scale. Phosphatidylcholine (PC), cholesterol (Chol), and cardiolipin (CL) molecules are shown as grey spheres.

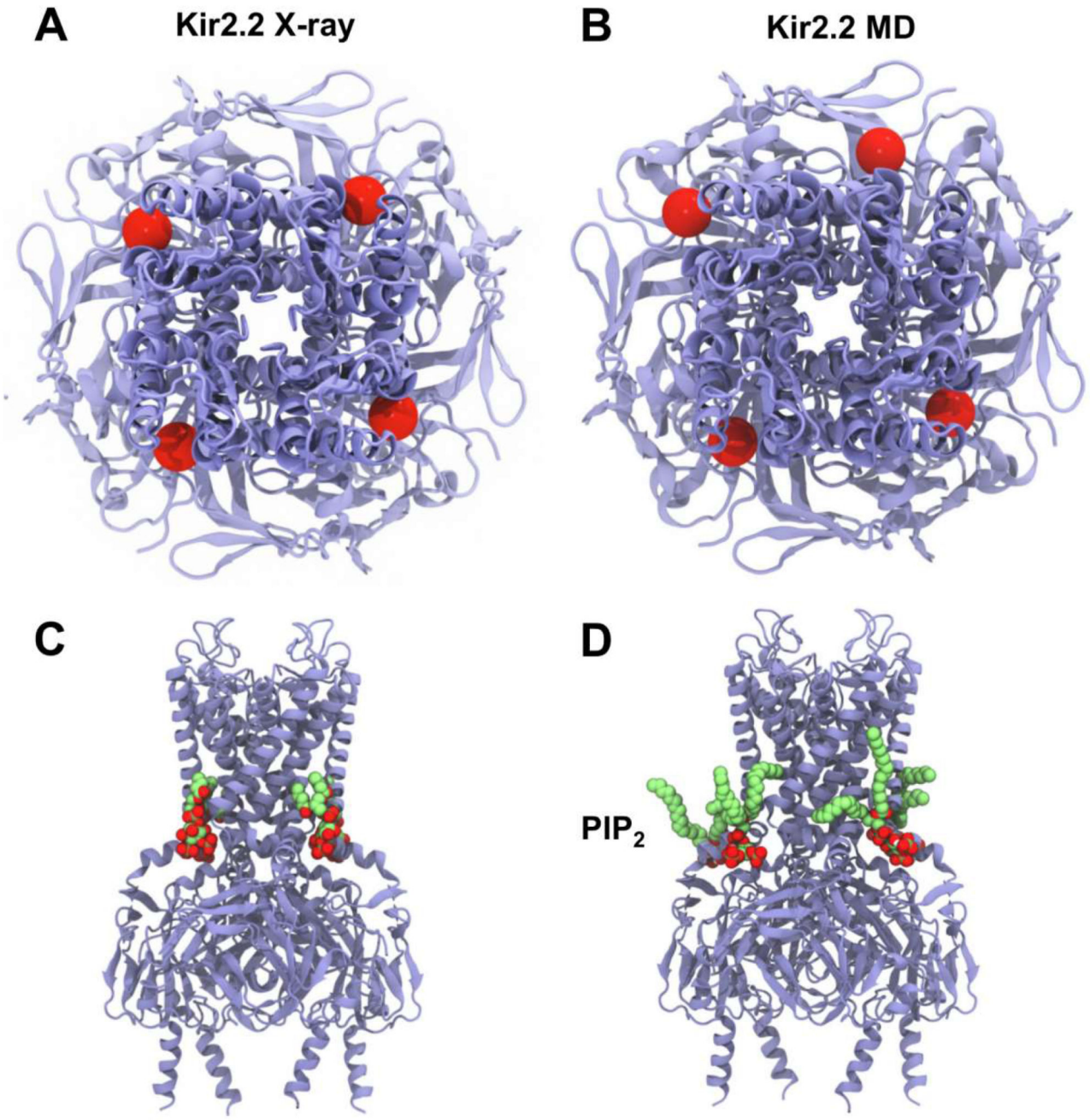

Figure 2.

Similarity in PIP_2 binding sites identified by crystallography [15] (A,C) and by simulation [25, 26] (B,D). (A, B) View from the extracellular side down the pore axis of the tetrameric Kir2.2 channel with the four PIP_2 α-phosphate groups (bridging the glycerol and inositol moieties) indicated as red spheres. (C, D) side view of the channel showing bound PIP_2 molecules (lime green carbons and red oxygens).

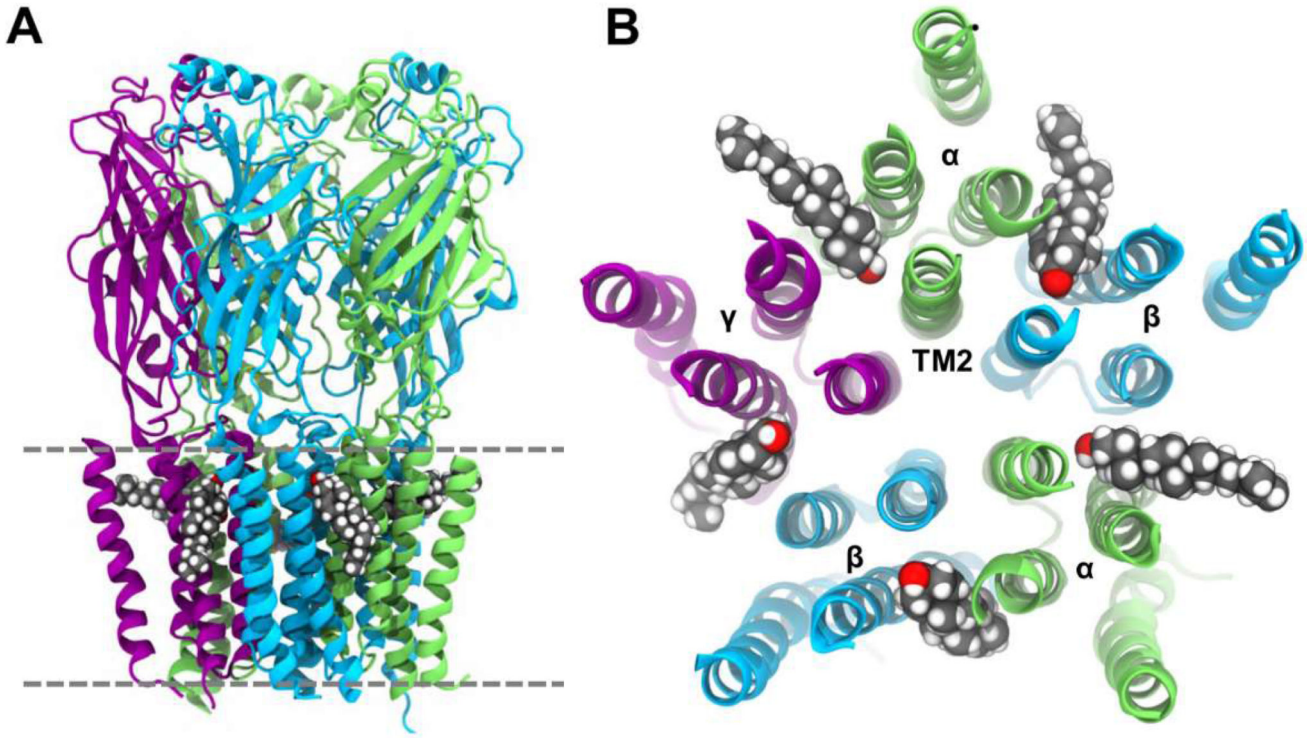

Figure 3.

Location of cholesterol binding sites between the five subunits of the pentameric $GABA_AR$ [63]. Initial coordinates were predicted *via* docking, and the structure shown is post-simulation. (A) sideview, and (B) extracellular view of the five transmembrane domains, with cholesterol molecules represented as van der Waals spheres. We thank Dr. Jérôme Hénin and Dr. Grace Brannigan for providing coordinates used to generate the figure.

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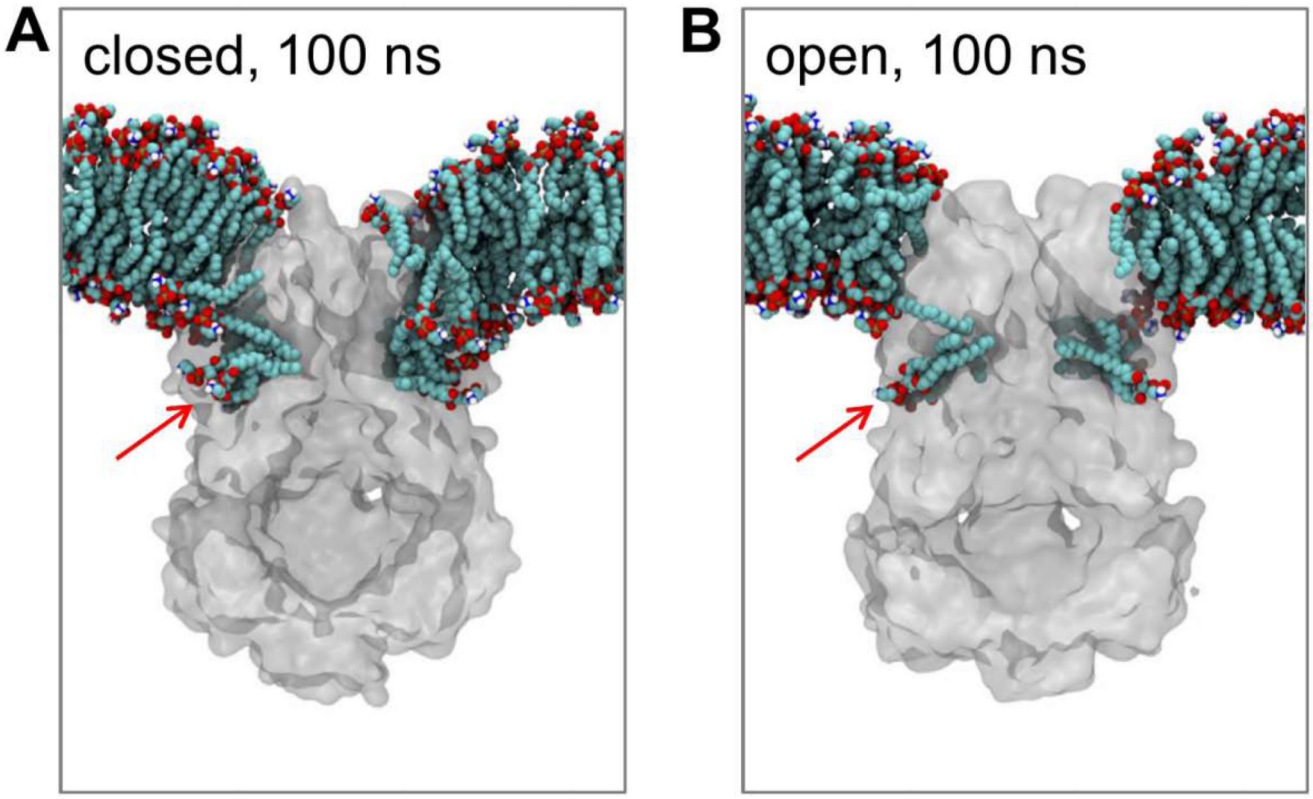

Figure 4.
MscS/lipid interactions comparing closed (PDB: 2OAU) and open (PDB: 5AJI) conformations. Cut-away slices are shown of snapshots at 100 ns from atomistic simulations of the closed (A) and open (B) conformation of MscS in POPE/POPG (4:1) bilayers [27]. The proteins are shown in grey and the lipids in cyan (carbons) and red (oxygens). The red arrow shows the lipid binding cavity whose occupancy changes between the closed and the open conformation. Figure modified from [27].

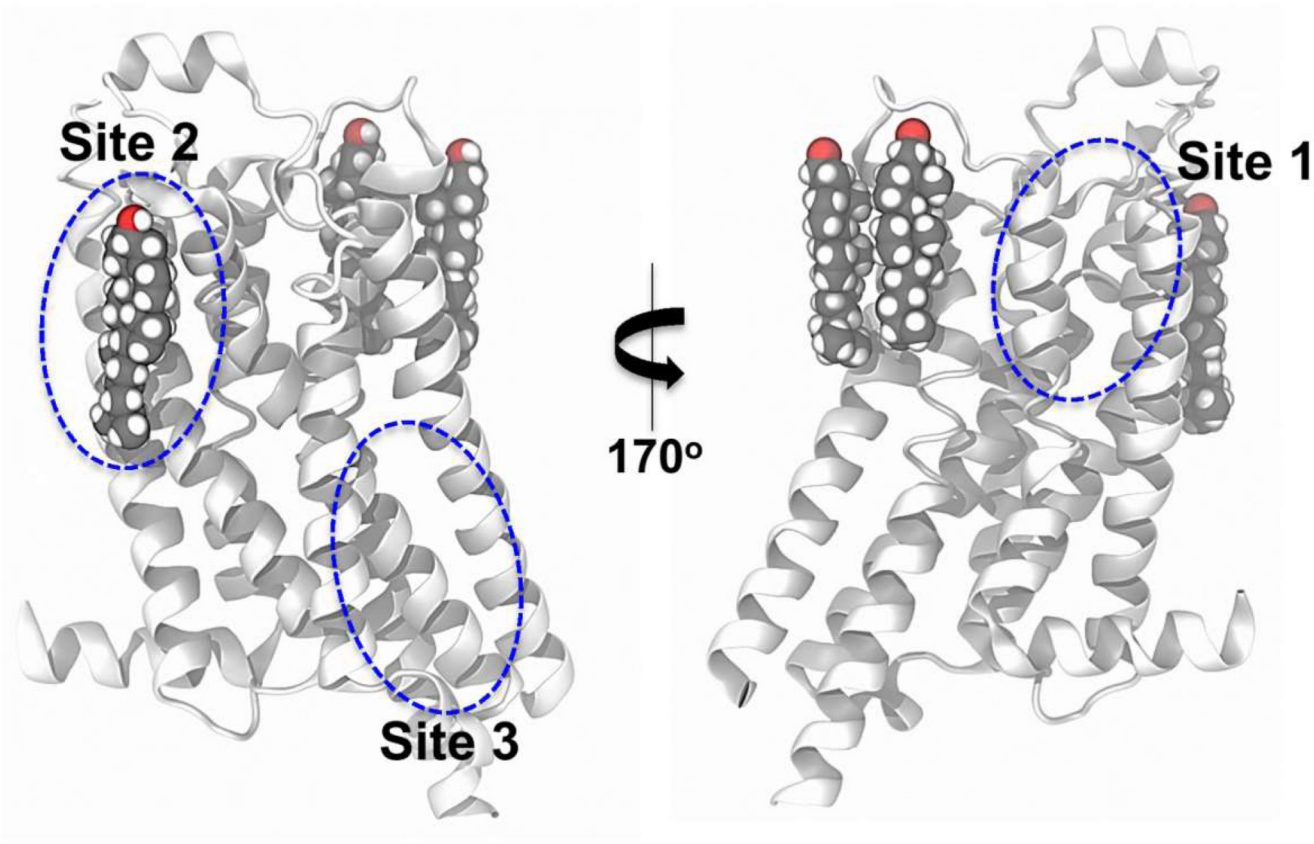

Figure 5.

Cholesterol interaction sites on the A_{2A} Adenosine receptor. (A) The location of cholesterol molecules (van der Waals spheres) resolved in a high resolution crystal structure of A_{2A} (PDB: 4EIY) are shown [90]. The approximate positions of cholesterol binding sites suggested by molecular simulation [24] are encircled in blue. Extracellular loop 2 is omitted for clarity.

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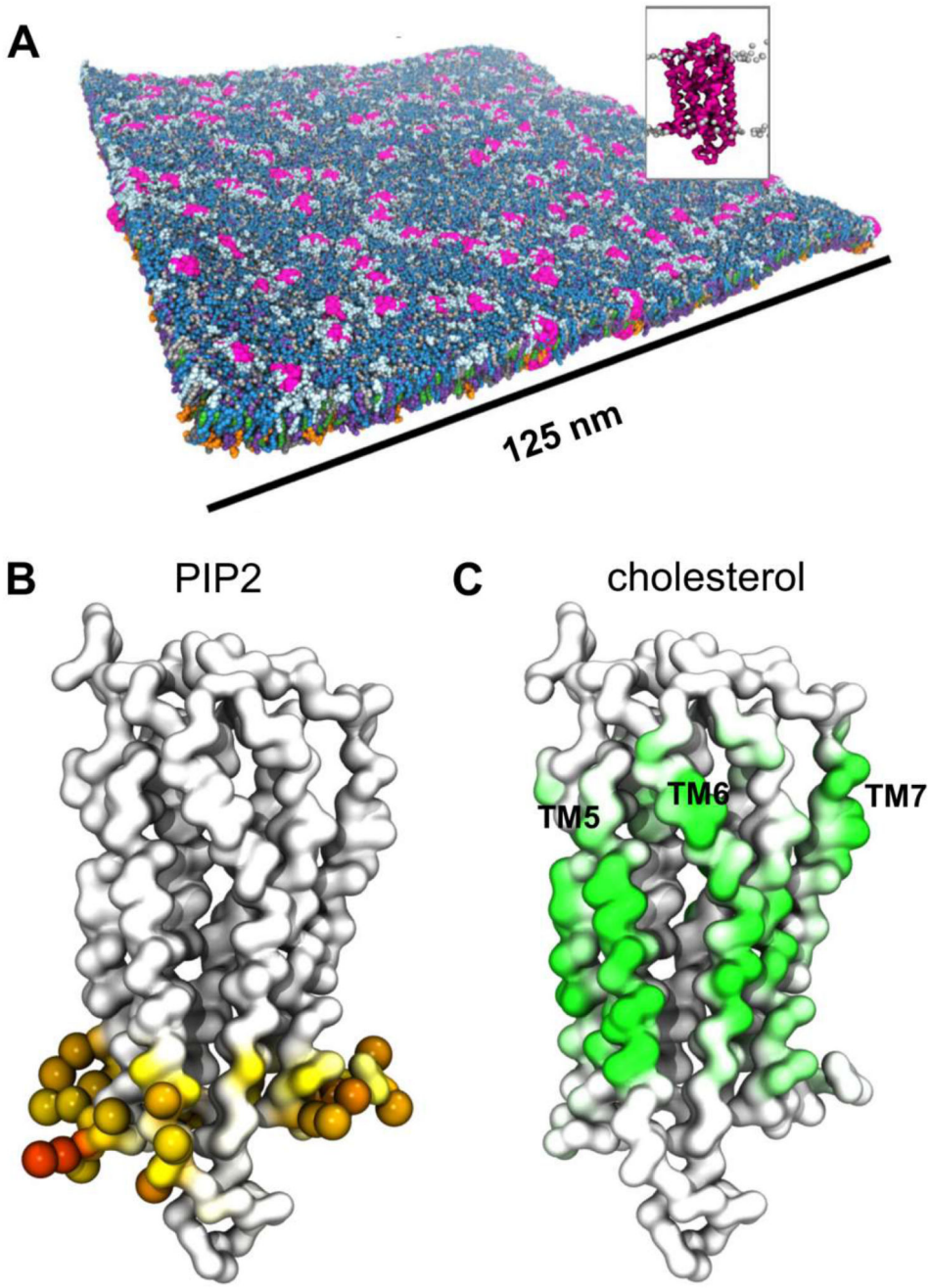

Figure 6.

Lipid interactions of the S1P1 receptor. (A) 144 S1P1 GPCR molecule (pink; see inset figure) embedded in a bilayer the lipid composition of which corresponds to that of a mammalian cell membrane. The image shown is from the end of a 10 μs CG-MD simulation [47]. (B) Model of the S1P1 receptor coloured according to the level of PIP_2 phosphoryl head group interaction, from white (no interaction) to orange (high interaction). Residues with high levels of PIP_2 interaction (>75% of simulation time) are shown as spheres. (C) The S1P1 receptor coloured according to the degree of cholesterol interaction from white

(no interaction) to green (high interaction). We thank Dr. Heidi Koldsø for the figure. Figure (adapted) reprinted with permission from [47]. Copyright (2015) American Chemical Society.

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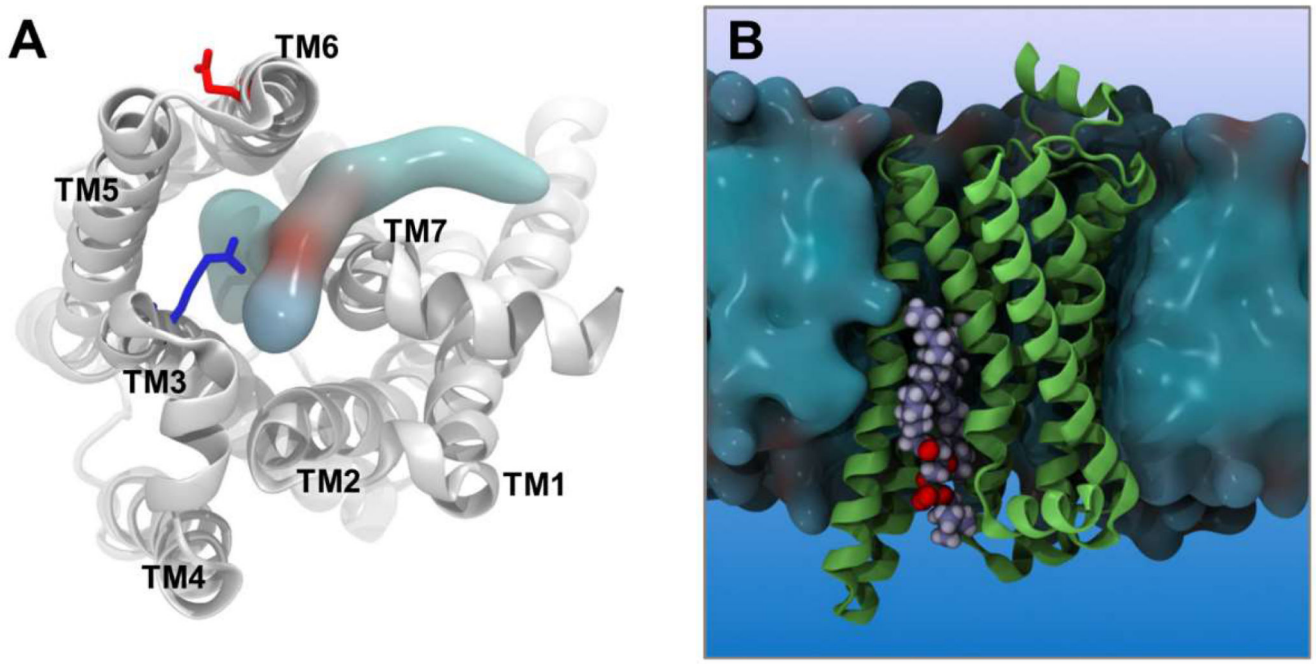

Figure 7.

PG/PC binding site within an activated state of the β_2AR [92]. (A) Penetration of a PC lipid into the core of the β_2AR. The residues R3.50 (blue) and E6.30 (red) of the ionic lock are indicated as sticks. (B) Side view of the receptor embedded in a lipid bilayer (blue surface) with a bound PC lipid (spheres, oxygens in red). We thank Dr. Chris Neale and Prof. Angel García for coordinates and simulation data used to produce the figure.

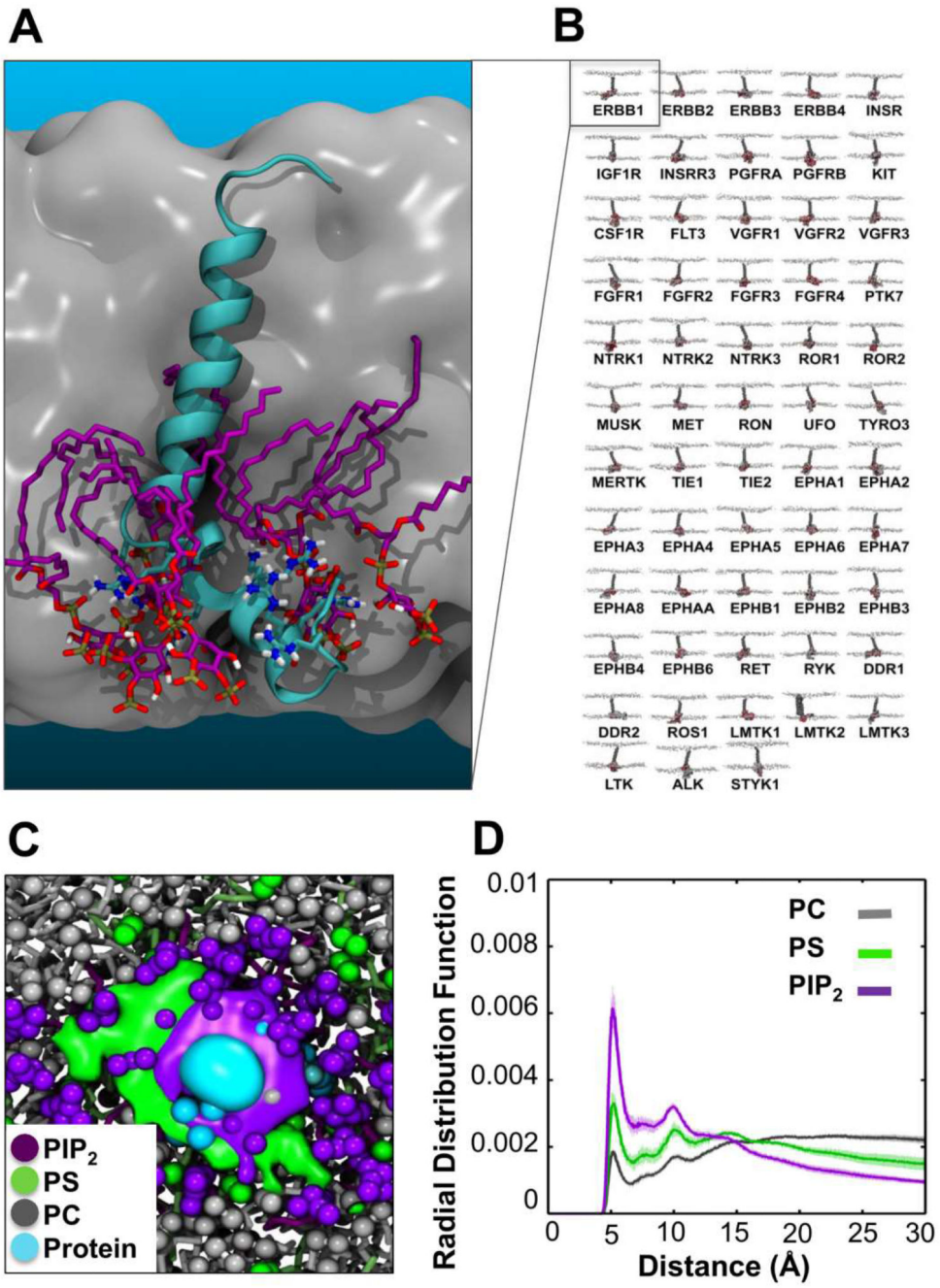

Figure 8.

Conserved interaction of PIP_2 lipids with TM-JM models of the human RTK superfamily [35]. (A) TM-JM model of the EGFR monomer (cyan) surrounded by a cluster of PIP_2 lipids (purple). (B) The 58 known members of the human RTK superfamily. (C) Clustering of anionic lipids around the monomeric TM-JM domain of the Insulin receptor. The image shows the average spatial occupancy of PIP_2 (purple), phosphatidylserine (green), and phosphatidylcholine (grey) lipids over the inner leafleft surface. The JM region density is

indicated in cyan. (D) Radial distribution function for each lipid species relative to the protein. Figure modified from [35].

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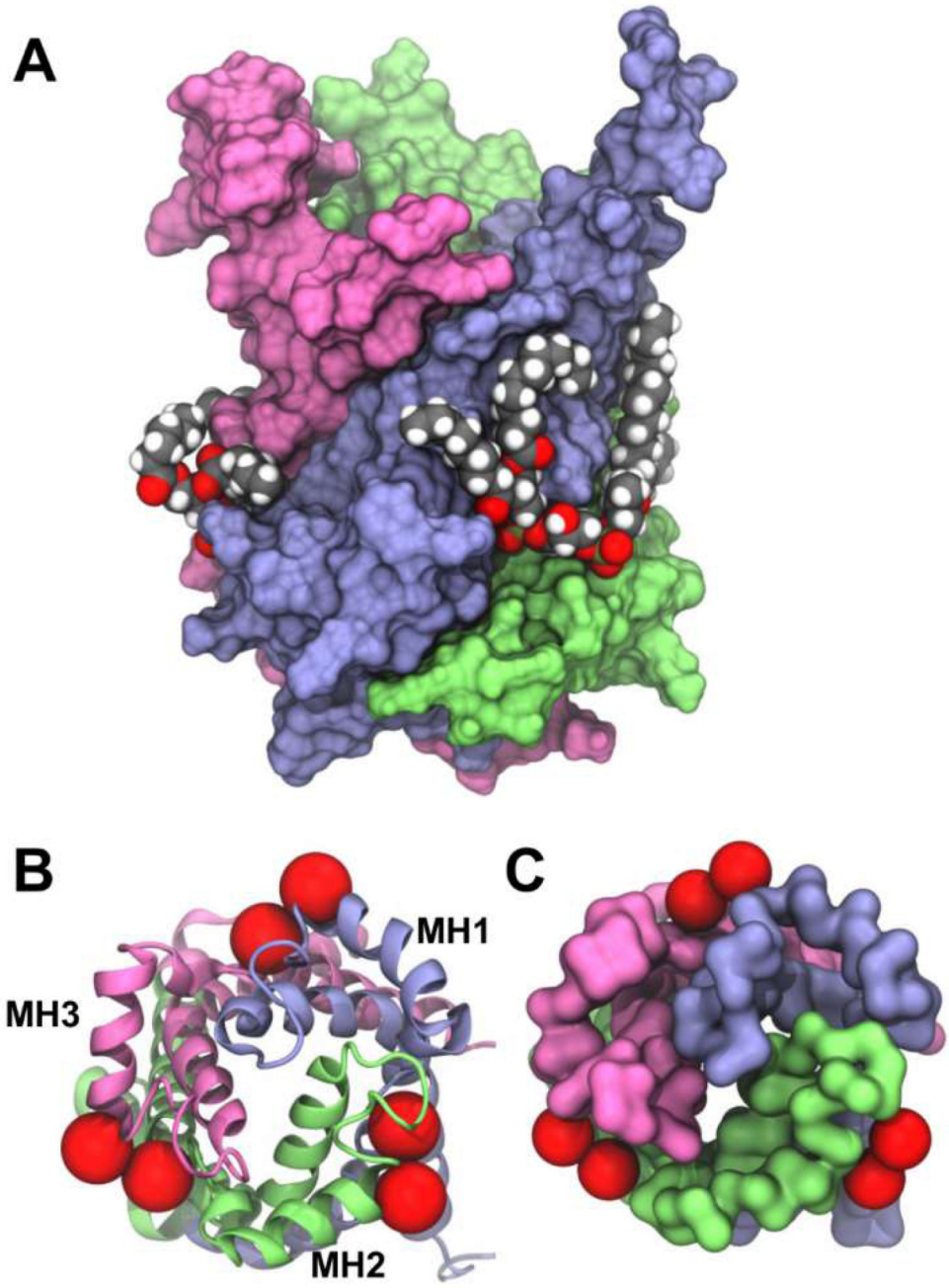

Figure 9.

The ADP/ATP carrier binds cardiolipin with a 1:3 stoichiometry at specific sites identified by experiment and CG simulation. (A) Side view of the bovine transporter (PDB: 2C3E) open to the intermembrane space, with crystallographically-resolved [130] cardiolipin molecules shown as van der Waals spheres. The protein surface is coloured according to its three fold pseudo-symmetry. (B) Crystallographic (PDB: 2C3E) and (C) simulation (*Rouse et al., unpublished data*) views onto the base of the transporter from the matrix. The approximate position of cardiolipin phosphate groups are indicated as red spheres (each

cardiolipin molecule contains two phosphate groups). The matrix-facing helices at the bilayer surface are labelled MH1 to MH3.

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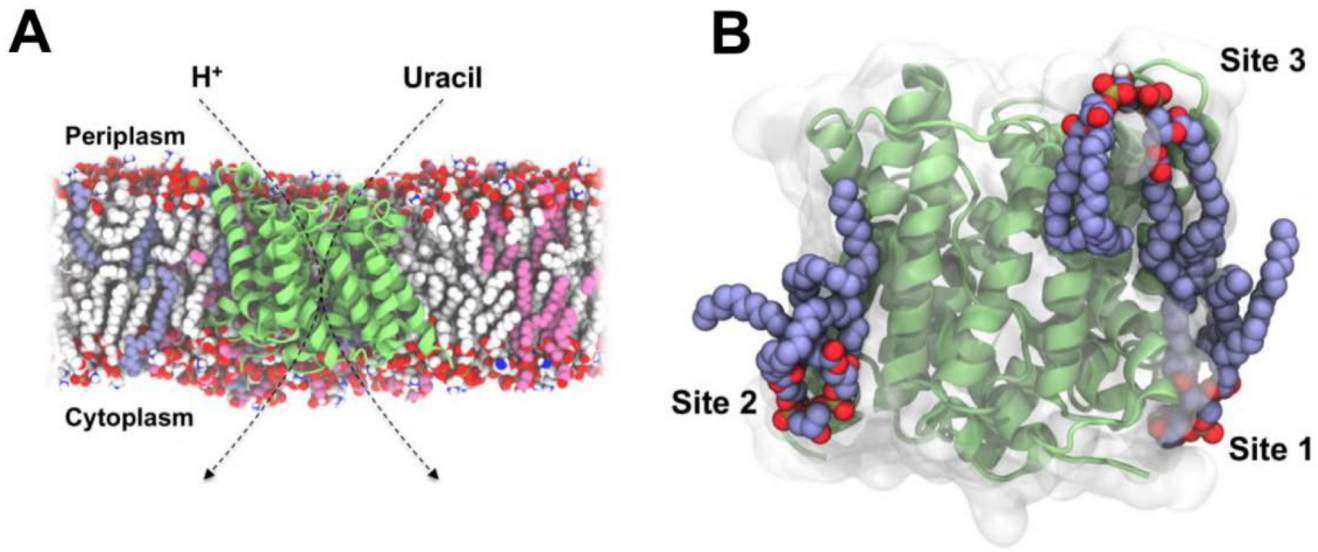

Figure 10.

Cardiolipin binding sites on the UraA H^+-Uracil symporter [60]. (A) Cross-section through a model of an *E. coli* inner membrane containing UraA (lime), PE (white), PG (pink), and cardiolipin (blue). (B) A representative simulation snapshot showing three predicted cardiolipin binding sites on the transporter. We thank Dr. Antreas Kalli for coordinates used to produce the figure.

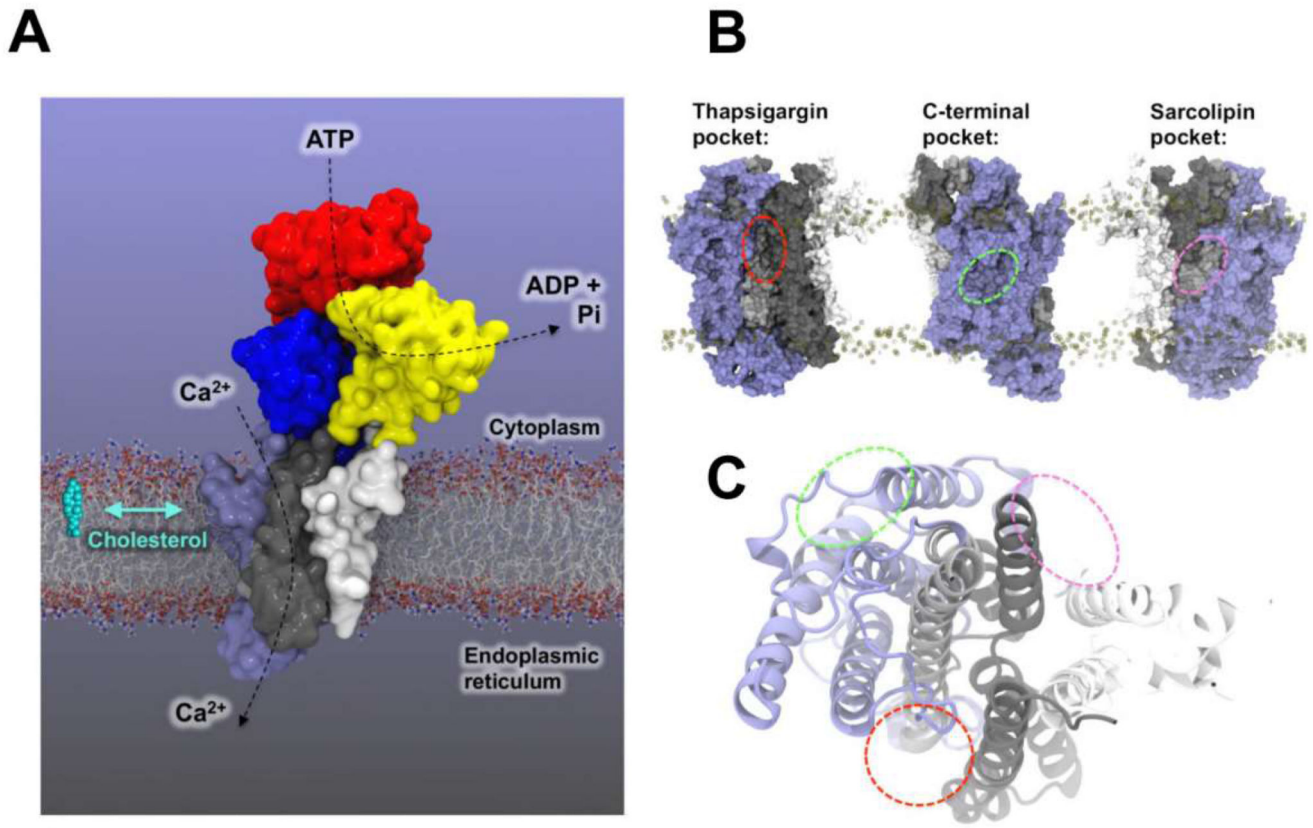

Figure 11.

SERCA architecture and putative cholesterol binding pockets. (A) Architecture and domain organisation of SERCA in the E2 state (a simulated structure [126] derived from PDB 2C8K is shown). (B) Approximate locations of the thapsigargin (red line), C-terminal (green line), and sarcolipin (pink line) cholesterol binding pockets on the SERCA transmembrane domain (TM1-2: white, TM3-4: black, TM5-6: grey, TM7-10: blue). We are grateful to Dr. Henriette E. Autzen for providing coordinates for this diagram.